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13. ABSTRACT (Maximum 200 words) Disease has become an increasingly important issue for wildlife management considerations over the past two decades. Our long term goals are to understand the impacts of diseases on free-ranging tortoises in order to improve the sustainability of managed tortoise populations. One of our overall objectives is to improve the diagnosis of infectious diseases in tortoises. The specific objective of this project was to accumulate additional data on performance of the RAPTOR™ field-portable evanescent-wave biosensor for rapid diagnosis. Banked plasma samples were tested in a double-blind study under laboratory conditions, then from that data the parameters that define the reliability of a diagnostic test were estimated. Under the conditions described the RAPTOR™ was able to discriminate between true seropositive and true seronegative tortoise plasma. False positives were rare and false negatives were more frequent than false positives. Management Recommendations: When making tortoise management decisions on the basis of infectious disease diagnostics, it is critical to establish goals for the population of interest, to determine a necessary sample size to meet the goals for surveillance, and to consider the PPV and NPV of the tests before implementing any policy. The goals established for the tortoise population can help managers decide whether potential assay errors should impact decision-making, and whether the benefits of the field-portable format and lower per-sample cost of the RAPTOR™ assay outweigh its disadvantages in capital cost and International Traffic in Arms Regulations (ITAR) compliance.				
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Enclosure 1

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FINAL REPORT*

29 June 2007

BIOSENSOR FOR FIELD DIAGNOSTICS
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Enclosure 2

EXECUTIVE SUMMARY

Disease has become an increasingly important issue for wildlife management considerations over the past two decades. Our long term goals are to understand the impacts of diseases on free-ranging tortoises in order to improve the sustainability of managed tortoise populations. Adequate surveillance is fundamental for disease prevention and control, thus there is an increasing need for the development of diagnostic assays for wildlife management.

One of our overall objectives is to improve the diagnosis of infectious diseases in tortoises. The specific objective of this project was to accumulate additional data on performance of the RAPTOR™ field-portable evanescent-wave biosensor for rapid diagnosis. The biosensor is capable of detecting specific antibodies in tortoise plasma that reflect a history of exposure to *Mycoplasma agassizii*, which is a bacterial agent of upper respiratory tract disease suspected to have adverse effects on tortoise health at the population level. A standard protocol for using the biosensor and interpreting the test results was developed. Banked plasma samples were tested in a double-blind study under laboratory conditions, then from that data the parameters that define the reliability of a diagnostic test were estimated.

In this study the sensitivity of the RAPTOR™ (ability to identify exposed tortoises in the group of all exposed individuals) was 69%; the specificity (unexposed individuals with negative test result, out of all unexposed individuals tested) was 88%; the Positive Predictive Value (PPV: exposed individuals with positive test, out of all individuals with positive test) was 85%; and the Negative Predictive Value (NPV: unexposed individuals with negative test, out of all individuals with negative test) was 75%. Thus, on average under the conditions described the RAPTOR™ was able to discriminate between true seropositive and true seronegative tortoise plasma. False positives were rare and false negatives were a worse problem than false positives. For the samples tested in this study, the RAPTOR™ performed worse than in our pilot study conducted in 2003 (94%, 86%, 91%, and 88% sensitivity, specificity, PPV, and NPV, respectively), although still approaching the reliability of the standard laboratory-based ELISA obtained for many years for all parameters except sensitivity.

Management Recommendations: When making tortoise management decisions on the basis of infectious disease diagnostics, it is critical to establish goals for the population of interest, to determine a necessary sample size to meet the goals for surveillance, and to consider the PPV and NPV of the tests before implementing any policy. The PPV and NPV may be affected by the prevalence of disease in the population being studied. When conducting surveillance for exposure to *M. agassizii*, occasional false negative results from a population with high seroprevalence will likely not impact management decisions significantly. A single positive result from an adequately sampled population with low seroprevalence should be interpreted with caution, as it has a greater risk of being a false positive result. The goals established for the tortoise population can help managers decide whether such potential errors should impact decision-making, and whether the benefits of the field-portable format and lower per-sample cost of the RAPTOR™ assay outweigh its disadvantages in capital cost and International Traffic in Arms Regulations (ITAR) compliance.

INTRODUCTION

A potentially debilitating communicable upper respiratory tract disease (URTD; ref. 30) of desert tortoises (*Gopherus agassizii*) is thought to have contributed to population declines over parts of that species' natural ranges during the past two decades (2,7,16,17). The bacteria *Mycoplasma agassizii* and *Mycoplasma testudineum* naturally infect tortoises, and were shown by experimental infection studies of *G. agassizii* and *Gopherus polyphemus* tortoises to be etiologic agents of URTD (6,9,10,11). Mycoplasmosis of tortoises elicits an IgM antibody response approximately 4 weeks after exposure, which shifts to a long-lasting, predominantly IgY antibody response approximately 10 weeks after exposure (3). Re-exposure can further increase IgY antibody levels to a plateau. Serological monitoring therefore may be valuable for epidemiologic studies of mycoplasmal diseases of tortoises (5,8,19,20). Plasma from infected tortoises was used previously to develop a quantitative enzyme-linked immunosorbent assay (ELISA) for monitoring exposure to mycoplasma among free-ranging tortoises, and to aid decision-making to control the spread of mycoplasmal URTD (26,27). Tortoise conservation and recovery plans now formally include testing for URTD (25,29). Detection of specific antibodies may be used to diagnose infection and immune status of tortoises for decision making, especially with regard to management and conservation of legally protected species such as *Gopherus*. However, the ELISA and other laboratory-based assays require that plasma samples be kept cool in the field and shipped cold to a laboratory for testing. In practice, the minimum turnaround time from sample collection to data reporting can be several days, which is problematic to minimize the risk of spread of mycoplasmosis before results are obtained, and regarding the need for timely information for management decision making (5).

In an April, 2003 pilot study (4), we tested the feasibility of evanescent-wave biosensor technology to develop a field test for specific anti-*M. agassizii* antibodies in tortoise plasma. Briefly, the RAPTOR™ evanescent-wave biosensor is a laser-based polystyrene fiber optic sensor which can detect specific *G. agassizii* anti-mycoplasma antibody bound to mycoplasmal whole-cell lysate antigen. Under various experimental protocols, the signals from positive control tortoise plasma samples were up to seven times higher than the signals from negative control plasma samples, when using *M. agassizii* whole-cell lysate antigen-coated fiber optics and cyanine Cy5-labeled anti-tortoise immunoglobulin antibody HL673 developed in our laboratory (13). Comparative double-blind ELISA and RAPTOR™ assays of previously banked tortoise plasma samples for the presence of antibodies to *M. agassizii* were conducted, with the ELISA result as the expected outcome and the RAPTOR™ result as the observed outcome. Six samples in each of four categories (ELISA seronegative, low ELISA titer [1:64], mid-range ELISA titer [1:128], and high ELISA titer [1:512]) were assayed ($\chi^2 = 14.5$, $P < 0.0001$). The sensitivity (samples containing anti-*M. agassizii* antibody give a positive result), specificity (samples without anti-*M. agassizii* antibody give a negative result), positive predictive value (PPV: samples that give a positive result do contain anti-*M. agassizii* antibody), and negative predictive value (NPV: samples that give a negative result do not contain anti-*M. agassizii* antibody) of the RAPTOR™ assay were calculated from gold standard tortoise plasma samples traceable to tortoises experimentally inoculated with *M. agassizii*. The sensitivity, specificity, PPV, and NPV of RAPTOR™ vs. ELISA were 94% vs. 94%; 86% vs. 83%; 91% vs. 94%; and 88% vs. 83%, respectively. From those observations we concluded that the RAPTOR™ assay had sensitivity and PPV potentially equal to or better than ELISA. The specificity and NPV of

the RAPTOR™ assay compared favorably to ELISA. In a laboratory setting, the RAPTOR™ assay produced information equivalent to ELISA, with a protocol that could be performed in a few minutes by a minimally-trained technician. The per-sample cost of the RAPTOR™ assay was about 20% less than ELISA (excluding capital equipment costs for both assays), plus the field-portable RAPTOR™ assay has the potential to eliminate sample handling and express shipping costs. The specific objective of this project was to accumulate additional data on performance of the RAPTOR™ field-portable biosensor for rapid diagnosis of tortoise exposure to *M. agassizii*.

MATERIALS AND METHODS

RAPTOR™ Biosensor The RAPTOR™ (<http://www.resrchintl.com/raptor.html>) is a portable, four-channel fluorometric assay system that has been used for high-sensitivity monitoring of biological warfare agents, toxins, and other analytes (1,14,18,21,22), but never before used for seroepidemiology of wildlife. It represents the integration of optics, microfluidics, electronics, and software into a compact and rugged instrument for use in laboratory settings and field assays (24). The unit can automatically perform a user-defined, multi-step assay protocol while simultaneously tracking fluorescently-tagged chemical reactions occurring on the surface of each of the system's four disposable optical sensors (15,28). All fluids needed to perform an assay, with the exception of sample, are contained in the unit. The reagents are held in a pre-cooled phase-change module intended to keep each reagent at a temperature of 30 °C or less, minimizing deterioration of thermally-labile reagents. For this study, the RAPTOR™ (s/n 10044, loan of U.S. Marine Corps Natural Resources and Environmental Activities Division, Marine Air Ground Task Force Training Command, Twentynine Palms CA) was operated on a laboratory benchtop using line power, and controlled by connection to a Gateway E Series desktop computer using the Windows XP operating system and RAPTOR^{PLUS}™ version 3.0.04 build 2 software.

Waveguide Coating and Coupon Assembly The disposable polystyrene fiber optical sensors (waveguides; Research International cat. no. 2000-139-043-01) were handled carefully by their mounting flange using serrated 5-inch dressing forceps having tips covered with soft Tygon tubing (BioRad cat. no. 7318215). New waveguides were cleaned by washing in 100% ethanol or isopropanol for 2 min, followed by four 5-min rinses with water, and air drying. The distal tip of each waveguide was painted with flat black paint (Testor Acryl[®] 1370) to create an optical sink. *Mycoplasma agassizii* type strain PS6 (American Type Culture Collection [ATCC] cat. no. 700616) whole-cell lysate antigen was prepared in ATCC medium 988 supplemented with glucose and 20% v/v fetal bovine serum as described previously, and stored in polypropylene cryovials at -80 °C at a stock concentration of 200 µg protein/ml. The PS6 antigen was diluted 1:5 in phosphate-buffered normal saline, pH 7.2 (PBS) to a final coating concentration of 40 µg/ml. To coat the cleaned and painted waveguides to be used for specific antibody capture, individual waveguides were immersed up to the hub of the mounting flange in clean polypropylene 22 gauge, 1-inch hypodermic needle caps filled with approximately 500 µl PS6 antigen for either 2 hr at room temperature or overnight at 4 °C. As a positive control, waveguides were coated directly with tortoise plasma diluted 1:10 in PBS. As a negative control, waveguides were coated with SuperBlock (Pierce cat. no. 37537 per recommendation of

Research International), which is a buffered proprietary irrelevant protein solution used for blocking excess binding sites in immunoassays (www.piercenet.com). A separate disposable plastic assay coupon (Research International cat. no. 7100-115-205-02) containing four mounted waveguides was assembled for each specimen by the UV light-cured adhesive (Research International cat. no. 7100-115-202-01) procedure recommended by the manufacturer. Each coupon included two PS6-coated waveguides and positive and negative control waveguides.

Secondary Antibody Tortoise anti-*M. agassizii* antibodies bound to the PS6 antigen were detected with cyanine Cy5-labeled anti-tortoise immunoglobulin (Ig) mouse IgG monoclonal antibody HL673. Fresh aliquots of the HL673 were prepared by the University of Florida Hybridoma Core Facility as described previously (13). For conjugation to Cy5 (Amersham Biosciences cat. no. PA25000), the dye was added to 1 ml of HL673 (1 mg protein/ml) and incubated for 30 min, with agitation every 10 min. Labeled antibody was separated from excess unconjugated dye by low-pressure gel filtration chromatography through 16 x 60 mm Sephadex G-50 resin columns (GE Healthcare cat. no. PD-10) using PBS as the elution buffer. The fraction (approximately 1 ml collected) containing purified Cy5-labeled HL673 was stored at 4 °C. For RAPTOR™ assays, that stock solution was diluted 1:125 in PBS to a final concentration of approximately 8 µg/ml.

Tortoise Plasma Samples Banked (<1 yr in polypropylene cryovials in a -20 °C non-defrosting freezer) ELISA-seropositive and -seronegative lithium-heparinized plasma samples from free-ranging *Gopherus* tortoises previously analyzed by our laboratory were the reagent controls (12) used to explore various conditions of waveguide preparation and RAPTOR™ executable step programming (recipe) protocols. Plasma was diluted 1:10 in PBS for analysis. The minimum amount of plasma needed for each assay was 100 µl. Seropositive ($n = 16$) and seronegative ($n = 17$) samples were then assayed in a double-blinded format to estimate the parameters that define the reliability of the RAPTOR™ for this application (23).

Recipes and Assays Functioning of the RAPTOR™ is controlled by baseline (Appendix 1) and sample (Appendix 2) programming recipes. To begin an assay, a coupon is inserted into the RAPTOR™. The optics and microfluidics systems are then automatically activated. The instrument is calibrated by running the baseline recipe with 1 ml of PBS serving as a sham negative sample. Specimens were introduced through the injection port using a disposable beveled 20 gauge, 1.5-inch hypodermic needle fitted to a 5-cc disposable polypropylene syringe. Plasma was analyzed by running the sample recipe. Briefly, the injected tortoise plasma is pumped onto the PS6-coated antibody-capture (and positive and negative control) waveguides and incubated for 8 min, then the plasma is pumped out to waste and the waveguides are rinsed three times with wash buffer (8.3 mM phosphate buffer, pH 7.2, plus 0.05% Triton X-100). At this mark (Mark 1), any specific anti-*M. agassizii* antibodies that may have been present in the plasma have had a chance to accumulate on the antibody-capture waveguides. Specific anti-*M. agassizii* antibodies should not accumulate on the positive or negative control waveguides. The Cy5-labeled secondary antibody HL673 is then pumped onto the waveguides and incubated for 2 min, then the HL673 is pumped back to its reservoir and the coupon is re-filled with wash buffer. At this mark (Mark 5), if specific anti-*M. agassizii* antibodies were present in the plasma, then HL673 has had a chance to bind to them where they have accumulated on the antibody-capture waveguides. If no specific anti-*M. agassizii* antibodies were present in the plasma, then no

HL673 should bind to the antibody-capture waveguides. Also at this mark, the HL673 has had a chance to accumulate on the positive control waveguide coated with irrelevant tortoise Ig, but it should not accumulate on the SuperBlock-coated negative control waveguide. The difference (delta) between the laser-excited evanescent-wave Cy5 fluorescence at Mark 1 and Mark 5 is a measure of the amount of HL673 binding and thus reflects the presence or absence of specific anti-*M. agassizii* antibodies in the plasma. Excluding reagent setup and baseline calibration, a sample assay required about 15 min to complete, in contrast to 4-5 hr for the standard ELISA. The assay results (Appendix 3) for the four channels were stored in flash memory for download from the RAPTOR™ to a desktop computer.

Statistical Analyses The test interpretation was by comparison of the sample delta in two channels of the four-channel system to positive and negative controls, providing for duplicate measurements of each specimen. The delta was normalized as a percent of the fluorescence (pA) at sample recipe Mark 1 (delta%). After unblinding the samples, the effect of ELISA status, i.e. either negative or positive, on delta% was analyzed by one-way ANOVA using StatView 5.0.1 (SAS Institute, Cary NC). Although the effect was significant ($P < 0.05$), since the F-test for equality of variance showed that the variance of delta% was not equal for ELISA-negative and -positive samples (0.288 and 1.152, respectively; $F_{33/31 \text{ d.f.}} = 0.250$, $P < 0.001$; see Results), the non-parametric Mann-Whitney U test was used for post-hoc comparison. The cutoff between positive and negative delta% was determined retrospectively by inspection of relative frequency histograms of delta% for ELISA-negative and -positive samples. The sensitivity, specificity, PPV and NPV were then calculated as described (12,24).

RESULTS

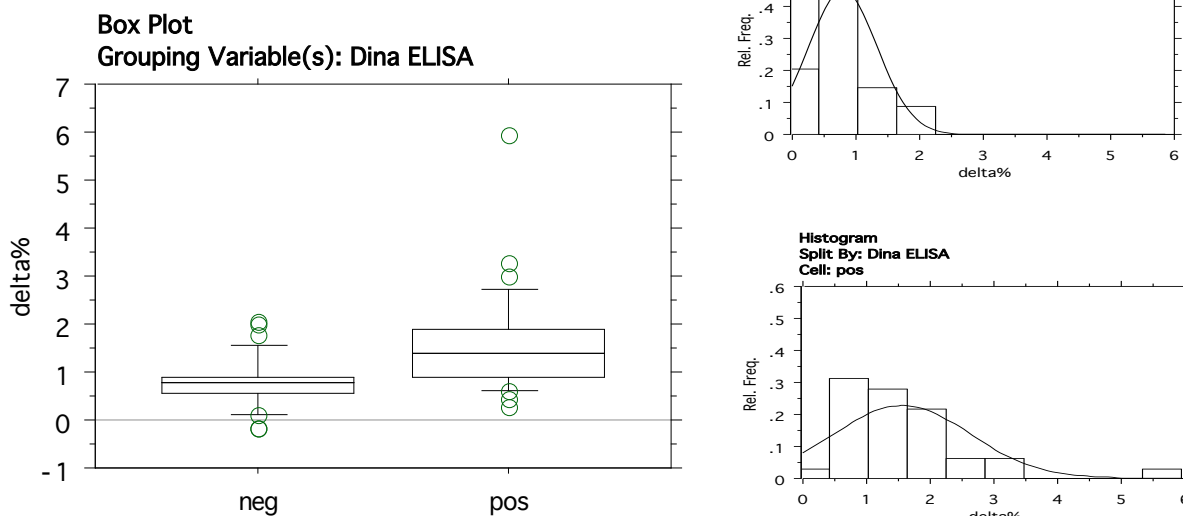
The mean delta% of ELISA-negative samples ($0.797 \pm \text{SE } 0.092$) was lower (Mann-Whitney $U = 249$; $P = 0.0002$) than that of ELISA-positive samples ($1.563 \pm \text{SE } 0.190$), thus on average under the conditions described the RAPTOR™ was able to discriminate between true seropositive and true seronegative tortoise plasma (Figure 1A). There was considerable overlap of the distribution of delta% of seropositive and seronegative plasma (Figure 1B), but inspection of the data revealed an obvious cutoff at delta% = 1 (Appendix 4). Using delta% < 1 = RAPTOR™-negative and delta% > 1 = RAPTOR™-positive, the sensitivity of the RAPTOR™ was 69%; the specificity was 88%; the PPV was 85%; and the NPV was 75%. Thus, in general, false positives were rare, and false negatives were a worse problem than false positives.

DISCUSSION

Detection of specific anti-mycoplasma antibodies can be used to diagnose infection and immune status of tortoises for epidemiology of natural populations, and for management decision-making to minimize the risk of spread of mycoplasmosis (5,31). The appearance of specific anti-*M. agassizii* antibodies in the plasma of tortoises can be detected reliably by quantitative ELISA 8 weeks after experimental inoculation with mycoplasma (26). There is a high positive correlation between presence of specific antibody against mycoplasma in tortoise plasma and URTD (27). Seropositive status is a significant risk factor for transmission of URTD (10,27). However,

laboratory-based assays for specific anti-mycoplasma antibody are limited by sample handling requirements, and by turnaround time which requires tortoise quarantine before decision making. We studied a field-portable RAPTOR™ test that might eliminate the current need for plasma sample refrigeration and shipping, and could provide nearly instant information for management decision making. The test could be performed in the field using a pre-programmed RAPTOR™ according to a standard protocol by personnel with no or limited training in immunoassay technology, and with a supply of consumables provided in the form of a kit.

Figure 1. RAPTOR™ delta% of *Mycoplasma agassizii* ELISA-negative and ELISA-positive tortoise plasma samples. **1A** (below): Box plot showing median, quartiles, and 90th percentiles. **1B** (right): Histograms of relative frequency.



The sensitivity of an immunoassay is the ability to identify exposed individuals in the group of exposed individuals, i.e., "if they were exposed, do they test positive?". It can only be estimated retrospectively by comparison to external standards, in this case the ELISA-validated serostatus. The 69% sensitivity, or 31% false-negative rate, obtained in the current study was considerably worse than the 94% sensitivity in our 2003 pilot study, and also worse than the standard ELISA (94%; ref. 31). Computationally, the difference is explained for most samples by consistently higher Mark 1 values, and consistently lower Mark 5 values, obtained in the current study. A comparatively large delta was necessary to reach the cutoff of delta% <1. Immunoassays in general are not precise enough to have a single cut point (31). Instead, a range that maximizes both sensitivity and specificity is usually necessary, but in this instance the sensitivity was not improved by adjusting the cutoff to any biologically meaningful extent. The difference in sensitivity was not attributable to differences between plasma sample sets in their specific antibody titers as measured by ELISA. Potential future approaches to minimizing the Mark 1 (background fluorescence) values include titering the antigen coating concentrations, incorporating a blocking step in the antibody-capture waveguide preparation protocol, and incorporating additional wash steps before Mark 1 in the sample recipe. Results obtained for the negative control waveguides in this study do not support future use of SuperBlock (Pierce cat. no. 37537) for blocking because of high background fluorescence. Potential approaches to maximizing the Mark 5 values include titering the Cy5 labeling protocol to increase the molar

ratio of dye bound to secondary antibody molecules, titering the working concentration of Cy5-labeled HL673, and increasing the length of incubation with Cy5-labeled HL673 before Mark 5 is taken. Unless it becomes commercially available in large quantities, lot-to-lot variation in the Cy5 labeling of HL673 seems likely to remain a significant source of variation in reliability of the RAPTOR™ assay.

The specificity of an immunoassay is the ability to identify unexposed individuals, i.e., "if they were not exposed, do they test negative?". This too can only be estimated retrospectively by comparison to external standards. The 88% specificity, or 12% false-positive rate, obtained in the current study was comparable to our pilot study (86%) and to the standard ELISA (86%). False positives actually were rare (only two samples, 14939 and 15094), and inspection of the raw data (Appendix 4) reveals that for each of those, one of the duplicate antigen-capture waveguides gave anomalous results. With any diagnostic test, some percentage of samples is expected to have test results that are false positive, false negative, or abnormal (12), but this result points out what we consider to be another of the most significant sources of variation in reliability of the RAPTOR™ system, which is its dependence on manual assembly of the delicate and optically fragile waveguides into coupons. Any flexion of, or physical contact with, the waveguide during coating or coupon assembly may lead to unreliable results. Sample 15281 for example was false-negative for similar reasons. Until waveguide coating and coupon assembly can be automated, this seems likely to remain an operator-dependent source of variation in reliability of the RAPTOR™ assay.

The PPV of an immunoassay is the ability to distinguish exposed individuals in a population of individuals with positive test results, i.e., "if they tested positive, were they really exposed?". It is used prospectively to make management decisions based on the test results. The 85% PPV obtained in the current study was slightly worse but approached the PPV of our pilot study (91%) and the standard ELISA (89-100%). The NPV of an immunoassay is the ability to distinguish unexposed individuals from exposed individuals that have a negative test result, i.e., "if they tested negative, were they really not exposed?". It too is used prospectively to make management decisions based on the test results. The 75% NPV obtained in the current study reflects the comparatively high false-negative rate, and was also slightly worse than the NPV in our pilot study (88%) and the standard ELISA (83-100%).

Since the ELISA for exposure to *M. agassizii* was first developed in 1992, a database of results from more than 20,000 tortoise samples has been generated. The ELISA was recently refined (31) by converting the reporting system from an optical density ratio to a titer-based system in order to make it more consistent with other serologic assays. Cutoff points were re-optimized and the corresponding Youden index was determined as a measure of the assay's diagnostic effectiveness. Further, more stringent quality assurance measures were incorporated to ensure optimum performance of the assay at all times. An adaptation of the Youden plot, which provides information pertaining to within-batch imprecision and drift as well as long term between batch reproducibility, was used for internal quality control. As modified, the ELISA PPV drops below 90% only at true seroprevalence <9%, and NPV drops below 90% at >85% true seroprevalence. For perspective, in the current sample set tested using the RAPTOR™ assay the true seroprevalence was 48%.

Future RAPTOR™ assay development recommendations: This study was first proposed in 2004 in response to needs expressed by the conservation community. The RAPTOR™ assay offered the major advantage that it could be developed for field seroepidemiology of almost any infectious disease of almost any species of conservation interest. Purchase of a RAPTOR™ biosensor dedicated for these experiments represented a major initial capital investment in this line of research. To date, our two studies have demonstrated that the RAPTOR™ biosensor is capable of detecting specific antibodies in tortoise plasma that reflect a history of exposure to *M. agassizii*, and that on average under the conditions described the RAPTOR™ was able to discriminate between true seropositive and true seronegative tortoise plasma. Two fundamental objectives remain: 1) assess the ability of the RAPTOR™ assay to discriminate tortoise antibodies to *M. agassizii* from tortoise antibodies to *Mycoplasma testudineum* or other potentially crossreactive antigens; and 2) assess the effects of environmental and sample-handling conditions likely to be encountered in the field on the performance characteristics of the RAPTOR™ assay. Those objectives represent the next essential stages of validation necessary before eventual field application to tortoise serodiagnostics would be justified. It is premature to expect the RAPTOR™ to be ready for scientifically-valid fieldwork at the current state of development. Because the technology remains promising, and because the capital equipment investment has been significant, the first reports from field trials are likely to have a substantial impact on public perception of the value of the technology for this application. The remaining objectives may be pursued in the future by any research team having access to a RAPTOR™ biosensor, relevant coating antigens, anti-tortoise Ig secondary antibodies, and externally-validated tortoise plasma controls.

United States Munitions List (USML) of International Traffic in Arms Regulations (ITAR) controlled articles: U.S. Department of State International Traffic in Arms Regulations (ITAR) apply to export of defense articles and services, including any technical data associated with such articles and services, that have been designed or modified for military use. The list of items regulated under ITAR is known as the U.S. Munitions List (USML). The term “export” as used in ITAR includes any: (1) actual shipment out of the U.S., or between foreign countries, of any covered goods or items; (2) the electronic or digital transmission out of the U.S., or between foreign countries, of any covered goods, items or related goods or items; or (3) any release or disclosure, including verbal disclosures or visual inspections, of any technology, software or technical data to any Foreign National/Person, even if the release occurs in the United States. The term “Foreign National/Person” means a person (natural person as well as a corporation, business association, partnership, society, trust, or any other entity, organization, or group, including government entities) who is not a lawful permanent resident of the U.S. An export may also include the actual use or application abroad of personal knowledge or technical experience acquired in the U.S. It recently came to our attention that the manufacturer of the RAPTOR™ system (Research International, Inc., Monroe WA; see <http://www.resrchintl.com/export.html>) has declared that the RAPTOR™ biosensor is subject to ITAR USML Category XIV export controls (see Appendix 5). Practical limitations on ability to assure security of an intentionally portable RAPTOR™ biosensor and to prevent export, as defined above, of the equipment (USML XIV*f) and related technical data (USML XIV*m) will likely constitute a previously unanticipated but now potentially substantial barrier to implementation of the RAPTOR™ in field situations as we originally envisaged.

MANAGEMENT RECOMMENDATIONS

When making tortoise management decisions on the basis of infectious disease diagnostics, it is critical to establish goals for the population of interest, to determine a necessary sample size to meet the goals for surveillance, and to consider the PPV and NPV of the tests before implementing any policy. For immunoassays, the PPV and NPV may be affected by the seroprevalence in the population being studied. Depending on the goals of the managers, it may be appropriate to shift assay cutoff points based on a desired sensitivity or specificity. Recent regulatory policies established by state and federal agencies have mandated serologic testing for *M. agassizii* exposure of tortoises impacted by human activities. Such policies have resulted in management decisions based solely on *M. agassizii* immunoassay results, including euthanasia of tortoises testing positive, without regard to the overall seroprevalence of the population and appropriate use of the assay. Given the potentially grave consequences for individual seropositive tortoises, for tortoise populations that include seropositive individuals, and or for introduction of infectious agents into environmentally-sensitive populations, managers may opt to maximize the specificity of the assay in order to reduce the probability of false-positive results. When conducting surveillance for exposure to *M. agassizii*, occasional false-negative results from a population with high seroprevalence will likely not impact management decisions significantly. A single positive result from an adequately sampled population with low seroprevalence should be interpreted with caution, as it has a greater risk of being a false-positive result. Further, the interpretation of test results and subsequent decision-making should be goal-oriented and based on a sound understanding of assay limitations. The goals established for the tortoise population can help managers decide whether potential errors should impact decision-making, and in this case whether the benefits of the field-portable format and lower per-sample cost of the RAPTOR™ assay outweigh its disadvantages in capital cost and International Traffic in Arms Regulations (ITAR) compliance.

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REFERENCES

1. Anderson, G.P., K.A. Breslin, and F.S. Ligler. 1996. Assay development for a portable fiber optic biosensor. *ASAIJ* 42:942-946.
2. Berry, K.H., D.R. Brown, M.B. Brown, E.R. Jacobson, J. Jarchow, J. Johnson, L.R. Richey, L.D. Wendland and R. Nathan. 2002. Reptilian mycoplasmal infections. *J. Herp. Med. Surg.* 12:8-20.
3. Brown, D.R. 2002. Mycoplasmosis and immunity of fish and reptiles. *Front. Biosci.* 7:1338-1346.

4. Brown, D.R., M.F. Kramer, L.A. Zacher, A.M. Green, and P.A. Klein. 2004. Chelonian serodiagnostics: development of a field portable assay for detection of exposure of tortoises to *Mycoplasma agassizii*. Desert Tortoise Council Symposium Proceedings (abstract).
5. Brown, D.R., I.M. Schumacher, G.S. McLaughlin, L.D. Wendland, M.B. Brown, P.A. Klein, and E.R. Jacobson. 2002. Application of diagnostic tests for mycoplasmal infections of desert and gopher tortoises, with management recommendations. *Chelonian Cons. Biol.* 4:497-507.
6. Brown, D.R., J.L. Merritt, E.R. Jacobson, P.A. Klein, J.G. Tully, and M.B. Brown. 2004. *Mycoplasma testudineum* sp. nov., isolated from desert tortoises (*Gopherus agassizii*) with upper respiratory tract disease. *Int. J. Syst. Evol. Microbiol.* 54:1527-1529.
7. Brown, D.R., L.A. Zacher, L.D. Wendland, and M.B. Brown. 2005. Emerging mycoplasmoses in wildlife. In *Mycoplasmas: pathogenesis, molecular biology, and emerging strategies for control*. Blanchard, A. and Browning, G., eds. Horizon Scientific Press, Norfolk U.K. pp. 383-414.
8. Brown, M.B., K.H. Berry, I.M. Schumacher, K.A. Nagy, M.M. Christopher, and P.A. Klein. 1999. Seroepidemiology of upper respiratory tract disease in the desert tortoise in the western Mojave Desert of California. *J. Wildl. Dis.* 35:716-727.
9. Brown, M.B., D.R. Brown, P.A. Klein, G.S. McLaughlin, I.M. Schumacher, E.R. Jacobson, H.P. Adams, and J.G. Tully. 2001. *Mycoplasma agassizii* sp. nov., isolated from the upper respiratory tract of the desert tortoise (*Gopherus agassizii*) and the gopher tortoise (*Gopherus polyphemus*). *Int. J. Syst. Evol. Microbiol.* 51:413-418.
10. Brown, M.B., G.S. McLaughlin, P.A. Klein, B.C. Crenshaw, I.M. Schumacher, D.R. Brown, and E.R. Jacobson. 1999. Upper respiratory tract disease in the gopher tortoise is caused by *Mycoplasma agassizii*. *J. Clin. Microbiol.* 37:2262-2269.
11. Brown, M.B., I.M. Schumacher, P.A. Klein, K. Harris, T. Correll, and E.R. Jacobson. 1994. *Mycoplasma agassizii* causes upper respiratory tract disease in the desert tortoise. *Infect. Immun.* 62:4580-4586.
12. Feldkamp, C.S. and J.L. Carey. 1997. Standardization of immunoassay methodologies. In N.R. Rose, E.C. de Macario, J.D. Folds, H.C. Lane, and R.M. Nakamura, eds. *Manual of Clinical Laboratory Immunology* (5th edition). American Society for Microbiology, Washington, D.C. p. 1168-1179.
13. Herbst, L.H., and P.A. Klein. 1995. Monoclonal antibodies for the measurement of class-specific antibody responses in the green turtle, *Chelonia mydas*. *Vet. Immunol. Immunopathol.* 46:317-335.
14. Hoyle, B. 2001. High-tech biosensor speeds bacteria detection. *Am. Soc. Microbiol. News* 9:434-435.
15. Hutchinson, A.M. 1995. Evanescent wave biosensors. Real-time analysis of biomolecular interactions. *Mol. Biotechnol.* 3:47-54.
16. Jacobson, E.R., J.M. Gaskin, M.B. Brown, R.K. Harris, C.H. Gardiner, J.L. LaPointe, H.P. Adams, and C. Reggiardo. 1991. Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). *J. Wildlife Dis.* 27:296-316.
17. Jacobson, E.R., M.B. Brown, I.M. Schumacher, B.R. Collins, R.K. Harris, and P.A. Klein. 1995. Mycoplasmosis and the desert tortoise (*Gopherus agassizii*) in Las Vegas Valley, Nevada. *Chelonian Cons. Biol.* 1:279-284.

18. Jung, C.C., E.W. Saaski, D.A. McCrae, B.M. Lingafelt, and G.P. Anderson. 2003. RAPTOR: a fluoroimmunoassay-based fiber optic sensor for detection of biological threats. *IEEE Sensors J.* 3:352-360.
19. Lance, V.A. 1994. Life in the slow lane: hormones, stress, and the immune system in reptiles. p. 529-534 *In* K.G. Davey, R.E. Peter, and S.S. Tobe (eds.), *Perspectives in Comparative Endocrinology*. National Research Council of Canada, Ottawa, Ontario.
20. Lance, V.A. Evaluating pain and stress in reptiles. 1992. p. 101-106 *In* D.O. Schaeffer, K.M. Kleinow, and L. Krulisch (eds.), *The Care and Use of Amphibians, Reptiles and Fish in Research*. Scientists Center for Animal Welfare, Bethesda, MD.
21. Lim, D.V. 2000. Rapid pathogen detection in the new millennium. *Nat. Food Proc. Assoc. J.* 2000:13-17.
22. Nath, N., S.R. Jain, and S. Anand. 1997. Evanescent wave fibre optic sensor for detection of *L. donovani* specific antibodies in sera of kala azar patients. *Biosens. Bioelectron.* 12:491-498.
23. National Committee for Clinical Laboratory Standards. 1994. Specifications for immunological testing for infectious diseases; approved guideline. NCCLS document I/LA18-A.
24. Ouellette, J. 1998. Biosensors: microelectronics marries biology. *Indust. Physic.* 3:11-14.
25. Savignano, D.A. 1996. The Clark County desert conservation plan. Desert Tortoise Council Symposium Proceedings (abstract).
26. Schumacher, I.M., M.B. Brown, E.R. Jacobson, B.R. Collins, and P.A. Klein. 1993. Detection of antibodies to a pathogenic mycoplasma in desert tortoises (*Gopherus agassizii*) with upper respiratory tract disease. *J. Clin. Microbiol.* 31:1454-1460.
27. Schumacher, I.M., D.B. Hardenbrook, M.B. Brown, E.R. Jacobson, and P.A. Klein. 1997. Relationship between clinical signs of upper respiratory tract disease and antibodies to *Mycoplasma agassizii* in desert tortoises from Las Vegas Valley, Nevada. *J. Wildlife Dis.* 33:261-266.
28. Taitt, C.R., G.P. Anderson, and F.S. Ligler. 2005. Evanescent wave fluorescence biosensors. *Biosens. Bioelectron.* 20:2470-2487.
29. U.S. Fish and Wildlife Service. 1994. Desert tortoise (Mojave population) Recovery Plan. U.S. Fish and Wildlife Service, Portland, Oregon. 73 pp. plus appendices.
30. Wendland, L.D., D.R. Brown, P.A. Klein, and M.B. Brown. 2006. Upper respiratory tract disease (mycoplasmosis) in tortoises. *In* *Reptile Medicine and Surgery* (2e). Mader, D.R., ed. Saunders Elsevier, St. Louis MO, pp. 931-938.
31. Wendland, L.D., L.A. Zacher, P.A. Klein, D.R. Brown, D. Demcovitz, R. Littell, and M.B. Brown. 2007. An improved ELISA for *Mycoplasma agassizii* exposure: A valuable tool in the management of environmentally sensitive tortoise populations. *Clin. Vaccine Immunol.* 14:(in press).

APPENDIX 1. Tortoise *Mycoplasma agassizii* RAPTOR™ Immunoassay Baseline Recipe.

<u>Command</u>	<u>Comment</u>
BufferPump Forward	
Wait 5	
SamplePump Forward	
Wait 16	
BufferPump Off	
SamplePump Off	
Wait 5	
AgitateSample 4	
Wait 12	
SamplePump Off	
SamplePump Forward	Load reagent to block
Wait 18	
ReagentPump Forward	
Wait 4	
SamplePump Off	
Wait 10	
WaitFor Fluid 7	
Laser 1111	
Wait 180	
Laser 0000	
ReagentPump Reverse	
Wait 18	
BufferPump Forward	
Wait 5	
ReagentPump Off	
Wait 3	
SamplePump Forward	
Wait 11	
BufferPump Off	
SamplePump Off	
Wait 10	
Mark	Mark=1 (use for initial wash data)
SamplePump Forward	
WaitFor air 22	Verifies coupon full of buffer and sample port empty
Laser 1111	
Wait 3	
LogData	
Wait 6	
HaltData	
Wait 1	
Laser 0000	
SamplePump Forward	
Wait 18	
ReagentPump Forward	Next 14 steps load reagents into coupon & collect data
Wait 4	
SamplePump Off	
ReagentPump Forward	
Wait 10	
WaitFor Fluid 7	Pumps reagents through coupon; verifies reagents loaded
Laser 1111	
Mark	Mark=2
LogData	Solution fluorescence data
Wait 3	Wait for signals to stabilize

Mark	Mark=3
Wait 6	Incubate 120 sec
HaltData	
Wait 1	
Laser 0000	Turn all four lasers off
Wait 20	
Laser 1111	
Wait 3	
LogData	30 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	
Wait 10	
LogData	60 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	
Wait 3	
LogData	90 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	
Wait 3	
LogData	
Wait 6	
HaltData	
Wait 1	
Laser 0000	
ReagentPump Reverse	
Wait 18	
BufferPump Forward	
Wait 5	
ReagentPump Off	
Wait 3	
SamplePump Forward	
Wait 9	
Mark	Mark=4
BufferPump Off	
SamplePump Forward	
WaitFor air 22	Verifies coupon full of buffer and sample port empty
Laser 1111	Turns all four lasers on
Wait 3	Warm up lasers
Mark	Mark=5
LogData	
Wait 6	Logs 6 seconds of Mark 5 data, use to calculate wash delta
HaltData	
Wait 1	

Laser 0000
Wait 1
End

Turn all four lasers off

APPENDIX 2. Tortoise *Mycoplasma agassizii* RAPTOR™ Immunoassay Sample Recipe.

<u>Command</u>	<u>Comment</u>
SamplePump Forward	Next 9 steps load sample into coupon & incubate
Wait 10	
SamplePump Forward	
WaitFor Fluid 5	Pumps sample into coupon
SamplePump Forward	
WaitFor Air 15	
SamplePump Off	Verifies coupon full of sample and sample port empty
Wait 48	
AgitateSample 4	
Wait 12	Incubate sample
SamplePump Off	
Wait 48	
AgitateSample 4	2nd incubate
Wait 12	
SamplePump Off	
Wait 48	3rd incubate
AgitateSample 4	
Wait 12	
SamplePump Off	4th incubate
Wait 48	
AgitateSample 4	
Wait 12	5th incubate
SamplePump Off	
Wait 48	
AgitateSample 4	6th incubate
Wait 12	
SamplePump Off	
Wait 48	7th incubate
AgitateSample 4	
Wait 12	
SamplePump Off	Next 21 steps rinse sample port and coupon with buffer
Wait 48	
AgitateSample 4	
Wait 12	1st rinse of sample port
SamplePump Off	
Wait 48	
BufferPump Forward	2nd rinse of sample port
Wait 10	
BufferPump Off	
SamplePump Forward	2nd rinse of coupon, empties buffer into waste
Wait 10	
WaitFor air 10	
SamplePump Off	3rd rinse of sample port
BufferPump Forward	
Wait 4	
SamplePump Forward	Pumps 3rd rinse into coupon

Wait 8	
BufferPump Off	
WaitFor air 20	Verifies coupon full of buffer and sample port empty
Mark	Mark=1
Laser 1111	
Wait 3	
LogData	
Wait 6	
HaltData	
Wait 1	
Laser 0000	
SamplePump Forward	
Wait 19	Empties buffer into waste; fills coupon with air
ReagentPump Forward	Next 21 steps load reagents into coupon and collect data
Wait 4	
SamplePump Off	
ReagentPump Forward	
Wait 10	
WaitFor Fluid 7	Pumps reagents through coupon; verifies reagents loaded
ReagentPump Off	
Laser 1111	
Mark	Mark=2
Wait 2	
LogData	Solution fluorescence data
Wait 3	Wait for signals to stabilize
Mark	Mark=3; assay integral data
Wait 6	Incubate for binding data
HaltData	
Wait 1	Time for microprocessor to close data file
Laser 0000	Turn all four lasers off
Wait 20	
Laser 1111	
Wait 3	
LogData	30 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	
Wait 3	
LogData	60 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	
Wait 3	
LogData	90 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
Wait 20	
Laser 1111	

Wait 3	
LogData	120 sec
Wait 6	
HaltData	
Wait 1	
Laser 0000	
ReagentPump Reverse	Returns reagents to vials
Wait 18	
BufferPump Forward	Next steps rinse coupon; collect assay delta data
Wait 5	Fills sample port with 1 ml of buffer
ReagentPump Off	
Wait 3	
Mark	Mark=4
SamplePump Forward	
Wait 9	
BufferPump Off	
SamplePump Forward	
WaitFor air 22	Verifies coupon full of buffer and sample port empty
SamplePump Off	
Laser 1111	Turns all four lasers on
Wait 3	Warm up lasers
Mark	Mark=5
LogData	
Wait 6	Logs 6 seconds of assay delta data
HaltData	
Wait 1	Time for microprocessor to close data file
Laser 0000	Turn all four lasers off
Wait 1	
End	

APPENDIX 3. Example RAPTOR™ Sample Recipe Output.

Tortoise plasma sample 15210 (051007112.54)

Time (sec)	Channel 1 (pA)	Channel 2 (pA)	Channel 3 (pA)	Channel 4 (pA)	Temperature (°C)	Mark	Lasers	Amplifier Gain
536.51	821.7	2640.0	646.2	878.9	34.3	1	1111	1011
537.50	821.7	2640.0	648.3	879.4	34.2	1	1111	1011
538.49	821.7	2640.0	649.4	879.4	34.2	1	1111	1011
539.48	821.2	2640.0	649.9	879.4	34.2	1	1111	1011
540.52	821.2	2640.0	650.4	880.5	34.2	1	1111	1011
541.51	820.6	2640.0	650.9	879.4	34.1	1	1111	1011
582.48	2772.0	3679.5	2022.8	2006.0	34.4	2	1111	0011
583.47	2860.0	3685.0	2035.3	2012.1	34.3	2	1111	0011
584.52	2871.0	3696.0	2049.4	2016.7	34.3	2	1111	0011
585.51	2882.0	3696.0	2061.9	2019.8	34.2	3	1111	0011
586.49	2898.5	3701.5	2072.9	2022.9	34.2	3	1111	0011
587.48	2904.0	3696.0	2083.9	2026.0	34.2	3	1111	0011
588.53	2920.5	3707.0	2093.8	2028.6	34.2	3	1111	0011
589.51	2931.5	3707.0	2103.7	2031.6	34.2	3	1111	0011
590.50	2942.5	3707.0	2113.1	2033.2	34.2	3	1111	0011
615.49	3135.0	3751.0	2343.0	2093.8	34.4	3	1111	0001
616.48	3146.0	3751.0	2348.5	2092.8	34.3	3	1111	0001
617.47	3146.0	3751.0	2354.0	2091.2	34.3	3	1111	0001
618.52	3157.0	3751.0	2354.0	2091.2	34.3	3	1111	0001
619.50	3157.0	3751.0	2359.5	2092.2	34.3	3	1111	0001
620.49	3168.0	3751.0	2365.0	2093.3	34.3	3	1111	0001
645.48	3289.0	3789.5	2491.5	2211.0	34.6	3	1111	0000
646.47	3289.0	3784.0	2497.0	2205.5	34.6	3	1111	0000
647.52	3294.5	3784.0	2502.5	2205.5	34.6	3	1111	0000
648.50	3294.5	3784.0	2502.5	2205.5	34.6	3	1111	0000
649.49	3300.0	3789.5	2508.0	2205.5	34.6	3	1111	0000
650.48	3300.0	3789.5	2508.0	2211.0	34.6	3	1111	0000
675.47	3393.5	3806.0	2607.0	2238.5	34.7	3	1111	0000
676.52	3399.0	3811.5	2607.0	2238.5	34.7	3	1111	0000
677.51	3399.0	3806.0	2607.0	2238.5	34.7	3	1111	0000
678.49	3399.0	3806.0	2612.5	2244.0	34.7	3	1111	0000
679.48	3404.5	3811.5	2618.0	2249.5	34.7	3	1111	0000
680.47	3410.0	3811.5	2612.5	2244.0	34.7	3	1111	0000
705.52	3481.5	3828.0	2700.5	2266.0	34.8	3	1111	0000
706.51	3481.5	3828.0	2700.5	2271.5	34.7	3	1111	0000
708.48	3492.5	3833.5	2706.0	2277.0	34.7	3	1111	0000
709.47	3492.5	3828.0	2711.5	2277.0	34.7	3	1111	0000
710.52	3498.0	3833.5	2711.5	2282.5	34.7	3	1111	0000
711.50	3498.0	3833.5	2717.0	2282.5	34.7	3	1111	0000
773.46	2959.0	3322.0	2139.5	2777.5	34.9	5	1111	0000
774.50	2953.5	3311.0	2139.5	2777.5	34.9	5	1111	0000
775.49	2948.0	3311.0	2139.5	2777.5	34.9	5	1111	0000
776.48	2948.0	3305.5	2145.0	2777.5	34.9	5	1111	0000
777.47	2948.0	3305.5	2145.0	2777.5	34.9	5	1111	0000
778.51	2948.0	3300.0	2145.0	2777.5	34.8	5	1111	0000

COUPON ID = 1

ASSAY DATA FILE NAME = 05100712.54

ASSAY RESULT RESET ERROR LIMIT = 32000

BASELINE INTEGRAL ERROR LIMIT = -32000

AVERAGE MARK 5 FLARE LIGHT ERROR LIMIT = -32000

THIS DATA WAS TAKEN USING VERSION 1.38 OF RAPTOR.EXE

THE RAPTOR SERIAL NUMBER WAS UNKNOWN

THE RECIPE NAME WAS 'raptorj1.rcp'

CHANNEL 1 STRING WAS 'PS6'

CHANNEL 2 STRING WAS 'PLASMA POS C'

CHANNEL 3 STRING WAS 'PS6'
CHANNEL 4 STRING WAS 'PIERCE BLOCK'

AssaysRemaining = 0

AssayPauseTime = 0 s

CHANNEL 1: NORMALIZED INTEGRAL WAS = 334.515 (pA)
CHANNEL 1: ASSAY INTEGRAL WAS = 381.847 (pA)
CHANNEL 1: BASELINE INTEGRAL WAS = 47.332 (pA)
CHANNEL 1: NEW BASELINE IS = 381.847 (pA)
CHANNEL 1: NORMALIZED DELTA WAS = -212.667 (pA)
CHANNEL 1: PREVIOUS DELTA WAS = 358.417 (pA)
CHANNEL 1: AVERAGE PREVIOUS MARK5 DATA WAS = 2805.000 (pA)
CHANNEL 1: AVERAGE MARK1 DATA WAS = 821.325 (pA)
CHANNEL 1: AVERAGE MARK2 DATA WAS = 2834.333 (pA)
CHANNEL 1: AVERAGE MARK5 DATA WAS = 2950.750 (pA)
CHANNEL 1: SCALING FACTOR WAS = 1.000
CHANNEL 1: SUSPECT INTEGRAL LIMIT WAS = 10.000 (pA)
CHANNEL 1: POSITIVE INTEGRAL LIMIT WAS = 30.000 (pA)
CHANNEL 1: HIGH POSITIVE INTEGRAL LIMIT WAS = 100.000 (pA)
CHANNEL 1: SUSPECT DELTA LIMIT WAS = 10.000 (pA)
CHANNEL 1: POSITIVE DELTA LIMIT WAS = 30.000 (pA)
CHANNEL 1: HIGH POSITIVE DELTA LIMIT WAS = 100.000 (pA)
CHANNEL 2: NORMALIZED INTEGRAL WAS = -268.442 (pA)
CHANNEL 2: ASSAY INTEGRAL WAS = 82.952 (pA)
CHANNEL 2: BASELINE INTEGRAL WAS = 351.394 (pA)
CHANNEL 2: NEW BASELINE IS = 82.952 (pA)
CHANNEL 2: NORMALIZED DELTA WAS = -696.667 (pA)
CHANNEL 2: PREVIOUS DELTA WAS = 696.667 (pA)
CHANNEL 2: AVERAGE PREVIOUS MARK5 DATA WAS = 3973.750 (pA)
CHANNEL 2: AVERAGE MARK1 DATA WAS = 2640.000 (pA)
CHANNEL 2: AVERAGE MARK2 DATA WAS = 3686.833 (pA)
CHANNEL 2: AVERAGE MARK5 DATA WAS = 3309.167 (pA)
CHANNEL 2: SCALING FACTOR WAS = 1.000
CHANNEL 2: SUSPECT INTEGRAL LIMIT WAS = 10.000 (pA)
CHANNEL 2: POSITIVE INTEGRAL LIMIT WAS = 30.000 (pA)
CHANNEL 2: HIGH POSITIVE INTEGRAL LIMIT WAS = 100.000 (pA)
CHANNEL 2: SUSPECT DELTA LIMIT WAS = 10.000 (pA)
CHANNEL 2: POSITIVE DELTA LIMIT WAS = 30.000 (pA)
CHANNEL 2: HIGH POSITIVE DELTA LIMIT WAS = 100.000 (pA)
CHANNEL 3: NORMALIZED INTEGRAL WAS = 390.067 (pA)
CHANNEL 3: ASSAY INTEGRAL WAS = 404.626 (pA)
CHANNEL 3: BASELINE INTEGRAL WAS = 14.559 (pA)
CHANNEL 3: NEW BASELINE IS = 14.559 (pA)
CHANNEL 3: NORMALIZED DELTA WAS = 231.917 (pA)
CHANNEL 3: PREVIOUS DELTA WAS = 0.000 (pA)
CHANNEL 3: AVERAGE PREVIOUS MARK5 DATA WAS = 1910.333 (pA)
CHANNEL 3: AVERAGE MARK1 DATA WAS = 649.163 (pA)
CHANNEL 3: AVERAGE MARK2 DATA WAS = 2035.808 (pA)
CHANNEL 3: AVERAGE MARK5 DATA WAS = 2142.250 (pA)
CHANNEL 3: SCALING FACTOR WAS = 1.000
CHANNEL 3: SUSPECT INTEGRAL LIMIT WAS = 10.000 (pA)
CHANNEL 3: POSITIVE INTEGRAL LIMIT WAS = 30.000 (pA)
CHANNEL 3: HIGH POSITIVE INTEGRAL LIMIT WAS = 100.000 (pA)
CHANNEL 3: SUSPECT DELTA LIMIT WAS = 10.000 (pA)
CHANNEL 3: POSITIVE DELTA LIMIT WAS = 30.000 (pA)
CHANNEL 3: HIGH POSITIVE DELTA LIMIT WAS = 100.000 (pA)
CHANNEL 4: NORMALIZED INTEGRAL WAS = 118.821 (pA)
CHANNEL 4: ASSAY INTEGRAL WAS = 153.898 (pA)
CHANNEL 4: BASELINE INTEGRAL WAS = 35.076 (pA)
CHANNEL 4: NEW BASELINE IS = 35.076 (pA)
CHANNEL 4: NORMALIZED DELTA WAS = 797.758 (pA)
CHANNEL 4: PREVIOUS DELTA WAS = 260.442 (pA)

CHANNEL 4: AVERAGE PREVIOUS MARK5 DATA WAS = 1719.300 (pA)
CHANNEL 4: AVERAGE MARK1 DATA WAS = 879.508 (pA)
CHANNEL 4: AVERAGE MARK2 DATA WAS = 2011.592 (pA)
CHANNEL 4: AVERAGE MARK5 DATA WAS = 2777.500 (pA)
CHANNEL 4: SCALING FACTOR WAS = 1.000
CHANNEL 4: SUSPECT INTEGRAL LIMIT WAS = 10.000 (pA)
CHANNEL 4: POSITIVE INTEGRAL LIMIT WAS = 30.000 (pA)
CHANNEL 4: HIGH POSITIVE INTEGRAL LIMIT WAS = 100.000 (pA)
CHANNEL 4: SUSPECT DELTA LIMIT WAS = 10.000 (pA)
CHANNEL 4: POSITIVE DELTA LIMIT WAS = 30.000 (pA)
CHANNEL 4: HIGH POSITIVE DELTA LIMIT WAS = 100.000 (pA)

APPENDIX 4. Tortoise *Mycoplasma agassizii* RAPTOR™ Immunoassay Data.

Sample	Replicate	Mean Mark 1	Mean Mark 5	ELISA	Delta	Delta %	Mean Delta %
14939	1	718	2187	neg	1469	2.046	1.44
14939	2	694	1273	neg	579	0.834	.
14940	1	1364	2404	neg	1040	0.762	0.923
14940	2	748	2076	neg	1328	1.775	.
14943	1	904	1609	neg	705	0.78	0.761
14943	2	496	1216	neg	720	1.452	.
14951	1	612	1274	neg	662	1.082	0.953
14951	2	611	1114	neg	503	0.823	.
14961	1	488	1510	pos	1022	2.094	2.015
14961	2	415	1219	pos	804	1.937	.
14976	1	1016	1832	neg	816	0.803	0.781
14976	2	504	887	neg	383	0.76	.
14988	1	1068	1435	neg	367	0.344	0.461
14988	2	587	926	neg	339	0.578	.
15043	1	606	1401	pos	795	1.312	1.034
15043	2	739	1297	pos	558	0.755	.
15050	1	1289	2079	neg	790	0.613	0.662
15050	2	790	1352	neg	562	0.711	.
15083	1	1162	2153	neg	991	0.853	0.797
15083	2	767	1335	neg	568	0.741	.
15085	1	542	1337	neg	795	1.467	0.961
15085	2	487	708	neg	221	0.454	.
15093	1	1656	1844	neg	188	0.114	0.225
15093	2	1004	1340	neg	336	0.335	.
15094	1	612	1089	neg	477	0.779	1.399
15094	2	585	1766	neg	1181	2.019	.
15095	1	1001	1876	pos	875	0.874	1.274
15095	2	459	1228	pos	769	1.675	.
15105	1	745	1914	pos	1169	1.569	1.678
15105	2	607	1692	pos	1085	1.787	.
15115	1	899	1655	neg	756	0.841	0.868
15115	2	545	1032	neg	487	0.894	.
15121	1	667	1372	neg	705	1.057	0.577
15121	2	1192	1306	neg	114	0.096	.
15174	1	830	2570	pos	1740	2.096	1.48
15174	2	711	1325	pos	614	0.864	.
15184	1	2822	2395	neg	-427	-0.151	0.691
15184	2	751	1902	neg	1151	1.533	.
15187	1	620	1280	pos	660	1.065	0.671
15187	2	953	1217	pos	264	0.277	.
15188	1	1242	2271	pos	1029	0.829	0.743
15188	2	776	1285	pos	509	0.656	.
15189	1	1088	1923	neg	835	0.767	0.486
15189	2	941	1135	neg	194	0.206	.
15190	1	545	983	neg	438	0.804	0.676
15190	2	507	785	neg	278	0.548	.
15198	1	2917	2367	neg	-550	-0.189	0.192

15198	2	783	1231	neg	448	0.572	.
15206	1	722	5005	pos	4283	5.932	4.469
15206	2	520	2083	pos	1563	3.006	.
15210	1	821	2950	pos	2129	2.593	2.446
15210	2	649	2142	pos	1493	2.3	.
15233	1	661	1652	pos	991	1.499	1.433
15233	2	558	1321	pos	763	1.367	.
15239	1	3938	9815	pos	5877	1.492	2.378
15239	2	2034	8672	pos	6638	3.264	.
15281	1	2292	3314	pos	1022	0.446	0.769
15281	2	1015	2123	pos	1108	1.092	.
15282	1	953	2646	pos	1693	1.776	1.485
15282	2	576	1264	pos	688	1.194	.
15316	1	752	2076	pos	1324	1.761	1.569
15316	2	640	1522	pos	882	1.378	.
15328	1	635	1229	pos	594	0.935	0.498
15328	2	647	1049	pos	402	0.621	.
15337	1	1247	1996	pos	749	0.601	0.779
15337	2	634	1241	pos	607	0.957	.

APPENDIX 5. The United States Munitions List (USML) of International Traffic in Arms Regulations (ITAR)-controlled items. Category XIV (codified at 22 CFR Part 121).

Category XIV - Toxicological Agents, Including Chemical Agents, Biological Agents, and Associated Equipment

*(a) Chemical agents, to include:

(1) Nerve agents:

(i) O-Alkyl (equal to or less than C10, including cycloalkyl) alkyl (Methyl, Ethyl, n-Propyl or Isopropyl)phosphonofluoridates, such as: Sarin (GB): O-Isopropyl methylphosphonofluoridate (CAS 107-44-8) (CWC Schedule 1A); and Soman (GD): O-Pinacolyl methylphosphonofluoridate (CAS 96-64-0) (CWC Schedule 1A)

(ii) O-Alkyl (equal to or less than C10, including cycloalkyl) N,N-dialkyl (Methyl, Ethyl, n-Propyl or Iso-propyl)phosphoramidocyanidates, such as: Tabun (GA): O-Ethyl N, N-dimethylphosphoramidocyanidate (CAS 77- 81-6) (CWC Schedule 1A);

(iii) O-Alkyl (H or equal to or less than C10, including cycloalkyl) S-2-dialkyl (Methyl, Ethyl, n-Propyl or Isopropyl)aminoethyl alkyl (Methyl, Ethyl, n-Propyl or Iso propyl)phosphonothiolates and corresponding alkylated and protonated salts, such as: VX: O-Ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate (CAS 50782-69-9) (CWC Schedule 1A);

(2) Amiton: O,O-Diethyl S-[2(diethylamino)ethyl] phosphorothiolate and corresponding alkylated or protonated salts (CAS 78-53-5) (CWC Schedule 2A);

(3) Vesicant agents:

(i) Sulfur mustards, such as: 2-Chloroethylchloromethylsulfide (CAS 2625- 76-5) (CWC Schedule 1A); Bis(2-chloroethyl)sulfide (CAS 505-60-2) (CWC Schedule 1A); Bis(2-chloroethylthio)methane (CAS 63839-13-6) (CWC Schedule 1A); 1,2-bis (2-chloroethylthio)ethane (CAS 3563-36-8) (CWC Schedule 1A); 1,3-bis (2-chloroethylthio)-n-propane (CAS 63905-10-2) (CWC Schedule 1A); 1,4-bis (2-chloroethylthio)-n-butane (CWC Schedule 1A); 1,5-bis (2-chloroethylthio)-n-pentane (CWC Schedule 1A); Bis (2-chloroethylthiomethyl)ether (CWC Schedule 1A); Bis (2-chloroethylthioethyl)ether (CAS 63918-89-8) (CWC Schedule 1A);

(ii) Lewisites, such as: 2-chlorovinylldichloroarsine (CAS 541-25-3) (CWC Schedule 1A); Tris (2-chlorovinyl) arsine (CAS 40334-70-1) (CWC Schedule 1A); Bis (2-chlorovinyl) chloroarsine (CAS 40334-69-8) (CWC Schedule 1A);

(iii) Nitrogen mustards, such as: HN1: bis (2-chloroethyl) ethylamine (CAS 538-07-8) (CWC Schedule 1A); HN2: bis (2-chloroethyl) methylamine (CAS 51-75-2) (CWC Schedule 1A); HN3: tris (2-chloroethyl)amine (CAS 555- 77-1) (CWC Schedule 1A);

(iv) Ethyldichloroarsine (ED);

(v) Methyldichloroarsine (MD);

(4) Incapacitating agents, such as:

(i) 3-Quinuclidinyl benzilate (BZ) (CAS 6581-06-2) (CWC Schedule 2A);

(ii) Diphenylchloroarsine (DA) (CAS 712-48-1);

(iii) Diphenylcyanoarsine (DC);

***(b) Biological agents and biologically derived substances specifically developed, configured, adapted, or modified for the purpose of increasing their capability to produce casualties in humans or livestock, degrade equipment or damage crops.**

*(c) Chemical agent binary precursors and key precursors, as follows:

- (1) Alkyl (Methyl, Ethyl, n-Propyl or Isopropyl) phosphonyl difluorides, such as: DF: Methyl Phosphonyldifluoride (CAS 676-99-3) (CWC Schedule 1B); Methylphosphinyldifluoride;
- (2) O-Alkyl (H or equal to or less than C10, including cycloalkyl) O-2-dialkyl (methyl, ethyl, n-Propyl or isopropyl)aminoethyl alkyl (methyl, ethyl, N-propyl or isopropyl)phosphonite and corresponding alkylated and protonated salts, such as: QL: O-Ethyl-2-di-isopropylaminoethyl methylphosphonite (CAS 57856-11-8) (CWC Schedule 1B);
- (3) Chlorosarin: O-Isopropyl methylphosphonochloridate (CAS 1445-76-7) (CWC Schedule 1B);
- (4) Chlorosoman: O-Pinakolyl methylphosphonochloridate (CAS 7040-57-5) (CWC Schedule 1B);
- (5) DC: Methylphosphonyl dichloride (CAS 676-97-1) (CWC Schedule 2B); Methylphosphinyldichloride;

(d) Tear gases and riot control agents including:

- (1) Adamsite (Diphenylamine chloroarsine or DM) (CAS 578-94-9);
- (2) CA (Bromobenzyl cyanide) (CAS 5798-79-8);
- (3) CN (Phenylacetyl chloride or w-Chloroacetophenone) (CAS 532-27-4);
- (4) CR (Dibenz-(b,f)-1,4-oxazepine) (CAS 257-07-8);
- (5) CS (o-Chlorobenzylidenemalononitrile or o-Chlorobenzalmalononitrile) (CAS 2698- 41-1);
- (6) Dibromodimethyl ether (CAS 4497-29-4);
- (7) Dichlorodimethyl ether (ClCi) (CAS 542- 88-1);
- (8) Ethyldibromoarsine (CAS 683-43-2);
- (9) Bromo acetone;
- (10) Bromo methylethylketone;
- (11) Iodo acetone;
- (12) Phenylcarbylamine chloride;
- (13) Ethyl iodoacetate;

(e) Defoliants, as follows:

- (1) Agent Orange (2,4,5- Trichlorophenoxyacetic acid mixed with 2,4-dichlorophenoxyacetic acid);
- (2) LNF (Butyl 2-chloro-4-fluorophenoxyacetate)

***(f) Equipment and its components, parts, accessories, and attachments specifically designed or modified for military operations and compatibility with military equipment as follows:**

(1) The dissemination, dispersion or testing of the chemical agents, biological agents, tear gases and riot control agents, and defoliants listed in paragraphs (a), (b), (d), and (e), respectively, of this category;

(2) The detection, identification, warning or monitoring of the chemical agents and biological agents listed in paragraph (a) and (b) of this category;

(3) Sample collection and processing of the chemical agents and biological agents listed in paragraph (a) and (b) of this category;

(4) Individual protection against the chemical and biological agents listed in paragraphs (a) and (b) of this category.

(5) Collective protection against the chemical agents and biological agents listed in paragraph (a) and (b) of this category.

(6) Decontamination or remediation of the chemical agents and biological agents listed in paragraph (a) and (b) of this category.

(g) Antibodies, polynucleotides, biopolymers or biocatalysts specifically designed or modified for use with articles controlled in paragraph (f) of this category.

(h) Medical countermeasures, to include pre and posttreatments, vaccines, antidotes and medical diagnostics, specifically designed or modified for use with the chemical agents listed in paragraph (a) of this category and vaccines with the sole purpose of protecting against biological agents identified in paragraph (b) of this category. Examples include: barrier creams specifically designed to be applied to skin and personal equipment to protect against vesicant agents controlled in paragraph (a) of this category; atropine auto injectors specifically designed to counter nerve agent poisoning.

(i) Modeling or simulation tools specifically designed or modified for chemical or biological weapons design, development or employment. The concept of modeling and simulation includes software covered by paragraph (m) of this category specifically designed to reveal susceptibility or vulnerability to biological agents or materials listed in paragraph (b) of this category.

(j) Test facilities specifically designed or modified for the certification and qualification of articles controlled in paragraph (f) of this category.

(k) Equipment, components, parts, accessories, and attachments, exclusive of incinerators (including those which have specially designed waste supply systems and special handling facilities), specifically designed or modified for destruction of the chemical agents in paragraph (a) or the biological agents in paragraph (b) of this category. This destruction equipment includes facilities specifically designed or modified for destruction operations.

(l) Tooling and equipment specifically designed or modified for the production of articles controlled by paragraph (f) of this category.

(m) Technical data (as defined in Sec. 120.21 of this subchapter) and defense services (as defined in Sec. 120.8 of this subchapter) related to the defense articles enumerated in paragraphs (a) through (l) of this category. (See Sec. 125.4 of this subchapter for exemptions.) Technical data directly related to the manufacture or production of any defense articles enumerated elsewhere in this Category that are designated as Significant Military Equipment (SME) shall itself be designated as SME.

(n) The following interpretations explain and amplify the terms used in this category and elsewhere in this subchapter.

(1) A chemical agent in category XIV(a) is a substance having military application, which by its ordinary and direct chemical action, produces a powerful physiological effect.

(2) The biological agents or biologically derived substances in paragraph (b) of this category are those agents and substances capable of producing casualties in humans or livestock, degrading equipment or damaging crops and which have been modified for the specific purpose of increasing such effects. Examples of such modifications include increasing resistance to UV radiation or improving dissemination characteristics. This does not include modifications made only for civil applications (e.g., medical or environmental use).

(3) The destruction equipment controlled by this category related to biological agents in paragraph (b) is that equipment specifically designed to destroy only the agents identified in paragraph (b) of this category.

(4)

(i) The individual protection against the chemical and biological agents controlled by this category includes military protective clothing and masks, but not those items designed for domestic preparedness (e.g., civil defense). Domestic preparedness devices for individual protection that integrate components and parts identified in this subparagraph are licensed by the Department of Commerce when such components are:

(A) Integral to the device;

(B) inseparable from the device; and,

(C) incapable of replacement without compromising the effectiveness of the device.

(ii) Components and parts identified in this subparagraph exported for integration into domestic preparedness devices for individual protection are subject to the controls of the ITAR;

(5) Technical data and defense services in paragraph (m) include libraries, databases and algorithms specifically designed or modified for use with articles controlled in paragraph (f) of this category.

(6) The tooling and equipment covered by paragraph (l) of this category includes molds used to produce protective masks, overboots, and gloves controlled by paragraph (f) and leak detection equipment specifically designed to test filters controlled by paragraph (f) of this category.

(7) The resulting product of the combination of any controlled or non-controlled substance compounded or mixed with any item controlled by this subchapter is also subject to the controls of this category.

Note 1: This Category does not control formulations containing 1% or less CN or CS or individually packaged tear gases or riot control agents for personal self-defense purposes.

Note 2: Categories XIV(a) and (d) do not include the following:

(1) Cyanogen chloride;

(2) Hydrocyanic acid;

(3) Chlorine;

(4) Carbonyl chloride (Phosgene);

(5) Ethyl bromoacetate;

(6) Xylyl bromide;

(7) Benzyl bromide;

(8) Benzyl iodide;

(9) Chloro acetone;

- (10) Chloropicrin (trichloronitromethane);
- (11) Fluorine;
- (12) Liquid pepper.

Note 3: Chemical Abstract Service (CAS) registry numbers do not cover all the substances and mixtures controlled by this category. The numbers are provided as examples to assist the government agencies in the license review process and the exporter when completing their license application and export documentation.

Note 4: With respect to U.S. obligations under the Chemical Weapons Convention (CWC), refer to Chemical Weapons Convention Regulations (CWCER) (15 CFR parts 710 through 722). As appropriate, the CWC schedule is provided to assist the exporter.

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